

Bioprinting of Regenerative Photosynthetic Living Materials

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Living materials, which are fabricated by encapsulating living biological cells within a non-living matrix, have gained increasing attention in recent years. Their fabrication in spatially defined patterns that are mechanically robust is essential for their optimal functional performance but is difficult to achieve. Here, a bioprinting technique employing environmentally friendly chemistry to encapsulate microalgae within an alginate hydrogel matrix is reported. The bioprinted photosynthetic structures adopt pre-designed geometries at millimeter-scale resolution. A bacterial cellulose substrate confers exceptional advantages to this living material, including strength, toughness, flexibility, robustness, and retention of physical integrity against extreme physical distortions. The bioprinted materials possess sufficient mechanical strength to be self-standing, and can be detached and reattached onto different surfaces. Bioprinted materials can survive stably for a period of at least 3 days without nutrients, and their life can be further extended by transferring them to a fresh source of nutrients within this timeframe. These bioprints are regenerative, that is, they can be reused and expanded to print additional living materials. The fabrication of the bioprinted living materials can be readily up-scaled (up to ≥ 70 cm \times 20 cm), highlighting their potential product applications including artificial leaves, photosynthetic bio-garments, and adhesive labels.

1. Introduction

Living materials are bio-hybrid structures that are composed of biological living cells (either non-engineered or genetically engineered) housed within a non-living synthetic matrix, for example, organic or inorganic polymers, metals, ceramics, etc.^[1] The living cells in these materials endow them with novel functionalities such as sense-and-respond, energy production, production of high-value compounds, detoxification of harmful compounds, or self-healing capacities, among others.^[1a,2] Such functional living materials have been increasingly proposed for usage in potential applications including smart textiles, wearable devices, biosensors, or fermentation bioreactors.^[1b,2b,3b] However, controlling the spatiotemporal form of a living material, while endowing it with sufficient mechanical strength for the material to be self-standing, is hardly achievable.

3D printing has been shown to be an effective technology for the fabrication of living materials with controlled shapes and

sizes. Customized living materials from nano- to macroscales can be 3D printed with high resolution into spatially defined patterns.^[4] Materials and patterns can be designed with the aid of bioprinting such that they mimic the complex architecture, spatial organization, and time-evolving nature of living cells.^[5] Living cells from different taxonomic kingdoms (including algae, bacteria, fungi, yeast, plant, and animal cells) have been effectively bioprinted for fabrication of living functional materials.^[1b,6] Particularly, bioprinting of microalgae has gained considerable attention in the recent years.^[1b,7] Microalgae are biotechnologically profitable unicellular microorganisms that are capable of photosynthesis.^[7c,8] Due to their adaptability to harsh conditions, robustness, and sustainability, microalgae have been widely used in applications such as biofuel production, bioremediation, production of high-value metabolites (food and pharmaceutical grade), and wastewater treatment,^[7a,8b,9] and they have been printed into silk scaffolds capable of improving air quality.^[7c] Bioprinting of microalgae has been predominantly performed so far using scaffolds composed of natural, cell-friendly biopolymers such as alginate^[7b], carrageenan^[7a], silk^[7c], and starch.^[10] However, most of these bioprinted living materials remain fragile and lack mechanical strength; for example, the tensile strength of calcium alginate film ranges between ≈ 10 and 750 kPa,^[11] with toughness values ranging between ≈ 2 and 80 J m⁻³.^[12] The

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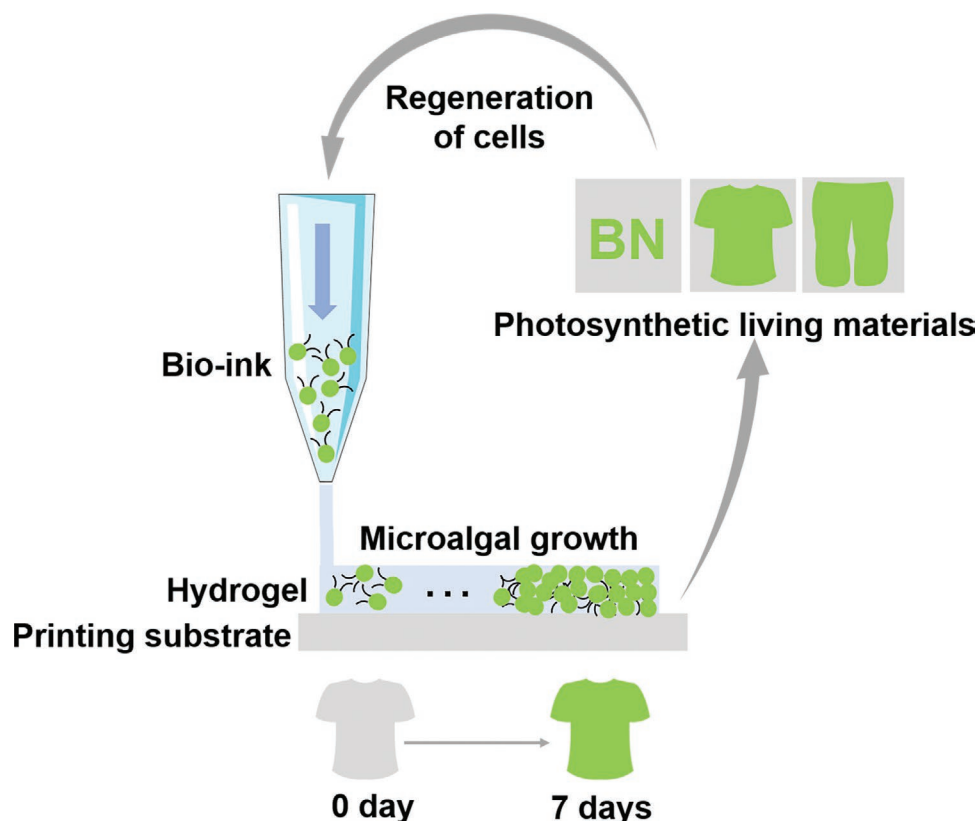


Figure 1. Bioprinting of photosynthetic living materials in a regenerative approach. Printing of a bio-ink (composed of sodium alginate and microalgae) onto a substrate (composed of bacterial cellulose and calcium chloride) results in the formation of an alginate hydrogel in which microalgal cells are immobilized. Placement of the bacterial cellulose overtop of a microalgal nutrient medium (minimal medium or carbon-supplemented medium) ensures microalgal growth within the bioprints on the bacterial cellulose over time. The bacterial cellulose supporting the living bioprinted microalgae can then be peeled off from the culture medium and used for various applications. Microalgal cells in the bioprints can also be regenerated and used as fresh bio-inks for further bioprinting processes.

fabrication of living materials that are self-standing and mechanically robust, while preserving the viability of encapsulated cells, remains a challenge.

Nature provides an inspiration for fabrication of such mechanically robust living materials.^[13] Materials present in nature possess better mechanical properties than the reported bioprinted living materials because of their hierarchical structure.^[14] In particular, cellulose produced by bacteria is a versatile, cell-friendly, and robust biopolymer^[15] with excellent tensile strength (73–194 MPa)^[15,16] and toughness (2–25 MJ m⁻³).^[15b,16,17] Bacterial cellulose is produced by the fermentation of bacteria such as *Gluconacetobacter hansenii* and *Komagataeibacter rhaeticus*.^[18] Bacterial cellulose has a nano-fibrous architecture and absorptive capabilities,^[19] which when used as a support for microalgal bioprints might allow nutrients to diffuse and reach the microalgal cells, thereby supporting their growth. Thus, we aimed to bioprint microalgae onto bacterial cellulose in order to combine the photosynthetic functionality of microalgae and the physico-mechanical properties of bacterial cellulose in the resultant living materials. Such photosynthetic self-standing living materials could be used in air purification by fixing carbon dioxide and releasing oxygen and waste water treatment by trapping heavy metals and pollutants.

In this study, we report a simple approach for the fabrication of living microalgal materials along with the development of cost-effective microalgal bioprinters. We employed a house-built bioprinter (Figure S1, Supporting Information) for patterning of microalgae onto agar and bacterial cellulose substrates (Figure 1). Here we show that microalgae are capable of being bioprinted as mono- or multi-layered constructs into various pre-defined geometries and sizes. Interestingly, the bioprints can be detached from the bacterial cellulose and re-attached to a fresh bacterial cellulose surface and retain adhesion to the new surface. These bioprinted microalgal structures are resilient to physical distortions and to immersion in water, indicating their physically stable nature. The bioprinted microalgal cells exhibit high viability over a period of at least 1 month. Furthermore, the patterned microalgae in the bioprints can be regenerated for preparation of fresh bio-inks. The microalgal cells bioprinted onto bacterial cellulose could survive stably at least 3 days following removal from nutrients, with their longevity being further extended when transferred onto fresh agar. Overall, these regenerative photosynthetic living materials of microalgae bioprinted overtop of bacterial cellulose offer diverse possibilities for novel product applications including artificial leaves, photosynthetic bio-garments, and adhesive labels.

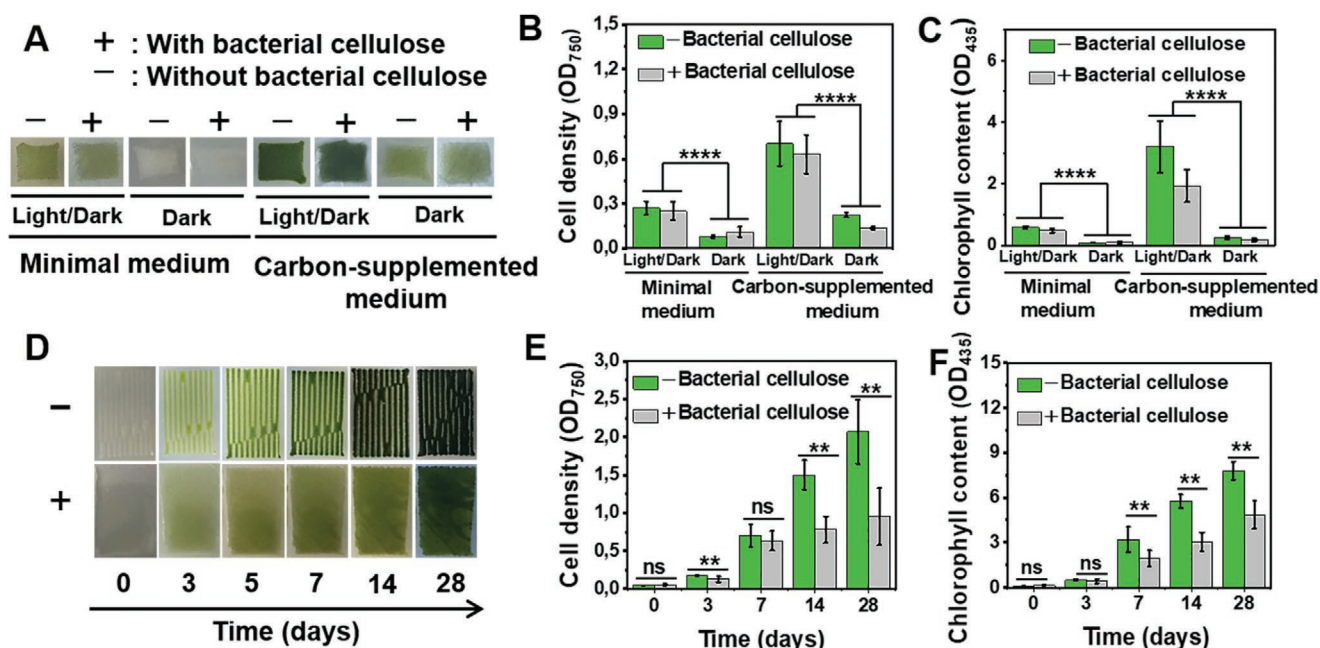


Figure 2. Optimal growth conditions and temporality of biprinted microalgal materials. Biprinted microalgae on agar (minimal medium and carbon-supplemented, indicated as “–bacterial cellulose”) and on bacterial cellulose overtop of agar (minimal medium and carbon-supplemented, indicated as “+ bacterial cellulose”) under light/dark and dark conditions (7 days of cultivation) were analyzed by A) photographs (–: printed directly on agar without bacterial cellulose; +: printed on bacterial cellulose overtop of agar), B) cell density measurement (O.D.₇₅₀), and C) chlorophyll content measurement (O.D.₄₃₅). Growth of biprinted microalgae over time was measured by D) photographs (–: printed directly onto agar without bacterial cellulose; +: printed onto bacterial cellulose overtop of agar), E) cell density measurements (O.D.₇₅₀), and F) chlorophyll measurements (O.D.₄₃₅) during cultivation under photomixotrophic conditions. ns, not significant ** $p < 0.01$ as determined by one-way (single factor) ANOVA with post-hoc Tukey’s HSD, **** $p < 0.0001$ as determined by two-way ANOVA.

2. Results

Living materials can be 3D printed by depositing living cells (bio-ink) onto a non-living matrix (substrate) in a layer-by-layer fashion. Both the bio-ink composition and the nature of the printing substrate play an important role in maintaining the viability and functionality of cells in the resultant biprinted materials as well as maintaining the overall 3D structure.

2.1. Optimal Growth Conditions for *Chlamydomonas reinhardtii* and Their Temporality in Biprinted Materials over Time

We investigated the growth of the microalgae *Chlamydomonas reinhardtii* on bacterial cellulose. *C. reinhardtii* can grow photoautotrophically (on CO₂ as a carbon source in the presence of light), chemotrophically/heterotrophically in the absence of light (on alternate carbon sources like acetate), or photomixotrophically (a combination of these two growth modes).^[20] In addition, cellulose has been demonstrated to be an alternative carbon source for the growth of *C. reinhardtii* under photomixotrophic conditions.^[21] However, the growth of microalgae on bacterial cellulose has not been evaluated so far. Thus, we evaluated the growth of microalgae on bacterial cellulose when placed overtop of carbon-supplemented agar and found that microalgae can grow on bacterial cellulose in photomixotrophic condition (Figure S2, Supporting Information).

We then determined the optimal conditions that yield the maximum microalgal cell growth and chlorophyll content within biprinted structures on agar (minimal or carbon-supplemented) or bacterial cellulose placed overtop of minimal or carbon-supplemented agar.

Bioprints grown photomixotrophically (Figure 2) either on agar (carbon-supplemented, light/dark condition) or on bacterial cellulose (overtop of carbon-supplemented agar, light/dark condition) had the most intense green coloration (Figure 2A) and yielded the maximum cell density (Figure 2B) and chlorophyll content (Figure 2C) in comparison with the photoautotrophic and chemotrophic conditions. Incubation under dark condition (minimal medium agar) yielded no visible growth in the samples, due to the absence of carbon source and light. Thus, the photomixotrophic growth condition was seen to be optimal for growth of microalgae in the bioprints and was used in all further experiments.

The viability of biprinted microalgal materials was investigated over time visually as well as by cell density (O.D.₇₅₀) and chlorophyll (O.D.₄₃₅) measurements at regular intervals of time for a total period of 4 weeks (Figure 2D–F). Immediately after printing (day 0), the calcium-alginate hydrogel matrix is transparent for samples both on carbon-supplemented agar or on bacterial cellulose overtop of carbon-supplemented agar. After 3 days of culturing, the green color of the microalgae was visible within the hydrogel matrix. Visual inspections indicated that the intensity of the green color within the bioprints increased over time (Figure 2D), consistent with results from microalgal cell density measurements, indicating a steady increase in

cell density (Figure 2E). For samples bioprinted onto agar, cell density within the bioprints increased steadily throughout the 28-day incubation period, whereas samples printed onto bacterial cellulose grew more slowly and reached maximum cell density after 7 days of incubation.

Chlorophyll content of the microalgal cells within the bioprints was observed by imaging the red fluorescence of chlorophyll induced upon UV excitation^[22] (Figure S3, Supporting Information) and by chlorophyll content measurement (Figure 2F). Chlorophyll content in the bioprinted microalgae increased by ≈ 101 -fold and ≈ 33 -fold (with respect to day 0) when grown upon carbon-supplemented agar and bacterial cellulose overtop of agar during 28 days of cultivation. The higher cell density and chlorophyll content of bioprinted microalgae on agar compared to bacterial cellulose overtop of agar is likely due to lesser availability of nutrients on bacterial cellulose. In total, our data show that microalgal cells survive the bioprinting process and are able to grow consistently within the bioprinted hydrogel matrices for at least a period of 4 weeks.

We additionally measured the viability of bioprinted microalgae after extended growth on either carbon-supplemented agar or on bacterial cellulose overtop of carbon-supplemented agar, by recovering and re-growing the microalgae from the bioprints. The microalgae were able to regrow in fresh carbon-supplemented medium even after 28 days of growth, indicating their long-term viability (Figure S4, Supporting Information).

We then assessed whether the bioprinted microalgae on bacterial cellulose would survive after removal from the nutrient source (carbon-supplemented agar). For this purpose, we first grew the bioprints on bacterial cellulose (overtop of carbon-supplemented agar) for 7 days under photomixotrophic condition. We then removed the bacterial cellulose containing the bioprinted microalgae from the carbon-supplemented agar and further incubated them under light/dark conditions. Cell density and chlorophyll measurements indicated that microalgae in the bioprints on bacterial cellulose could stably survive up to at least 3 days without contact with carbon-supplemented

agar, and their growth could be revived by placing them back onto fresh carbon-supplemented agar within 3 days of survival (Figure 3). To control for whether the process of removing the bacterial cellulose from carbon-supplemented agar affected the bioprinted microalgae, we measured the cell density of microalgae bioprints on bacterial cellulose that were lifted from the carbon-supplemented agar after 7 days, transferred to a fresh carbon-supplemented agar, and incubated for another 7 days. When compared to the cell density of a bioprint grown 14 days on the same carbon-supplemented agar, this transfer process was seen not to impede, but rather to increase the cell density of the bioprinted microalgae (Figure S5, Supporting Information). However, extended periods of incubation (≥ 5 days) without contact with carbon-supplemented agar led to an irreversible decrease in the microalgal cell density and chlorophyll content. Thus, the bioprinted living materials can survive for a period of at least 3 days after removal from nutrient source, and their longevity can be extended by placing them back onto a fresh nutrient source within this timeframe.

To assess whether there were morphological differences between the bioprinted and the planktonic microalgal cells, we performed scanning electron microscopy. The results (Figure S6, Supporting Information) revealed a uniform distribution of microalgal cells (with a mean cell diameter of 4–5 μm) with no morphological differences on both carbon-supplemented agar and bacterial cellulose overtop of carbon-supplemented agar. The observed cell sizes are consistent with previously reported cell sizes of *C. reinhardtii*.^[8b,23]

C. reinhardtii has been shown to utilize cellulose, which is composed of D-glucose units,^[24] as a carbon substrate via expression of endo β -1,4-glucanases. If the microalgae in the bioprints were to digest and degrade the bacterial cellulose substrate, the mechanical properties of the resulting material would be compromised and the material would be eventually degraded by the microalgae itself, which is undesirable. Hence, we assessed the cellulolytic activities of microalgae grown either planktonically in wells (8 mm in diameter) on carbon-supplemented agar

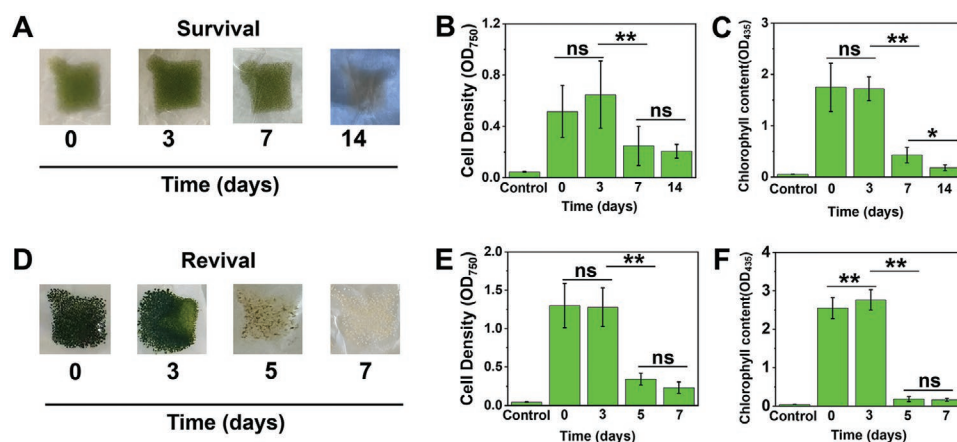


Figure 3. A–C) Survival and D–F) revival abilities of bioprinted microalgae material over time. Microalgal bioprints on bacterial cellulose were grown for 7 days under photomixotrophic conditions. The bioprints were assessed for survival after removal from carbon-supplemented agar for a total of 0, 3, 7, or 14 days. Their revival abilities were assessed by placing the microalgal bioprints back onto fresh carbon-supplemented agar for 7 days under photomixotrophic conditions. Bioprints were assessed via (A,D) photographs, (B,E) cell density (O.D.₇₅₀), and (C,F) chlorophyll (O.D.₄₃₅) measurements. Sterile sodium chloride (0.9 w/v%) and DMSO served as the controls in the (B,E) cell density and (C,F) chlorophyll measurements, respectively. ns, not significant * $p < 0.05$, ** $p < 0.01$ as determined by one-way (single factor) ANOVA with post-hoc Tukey's HSD.

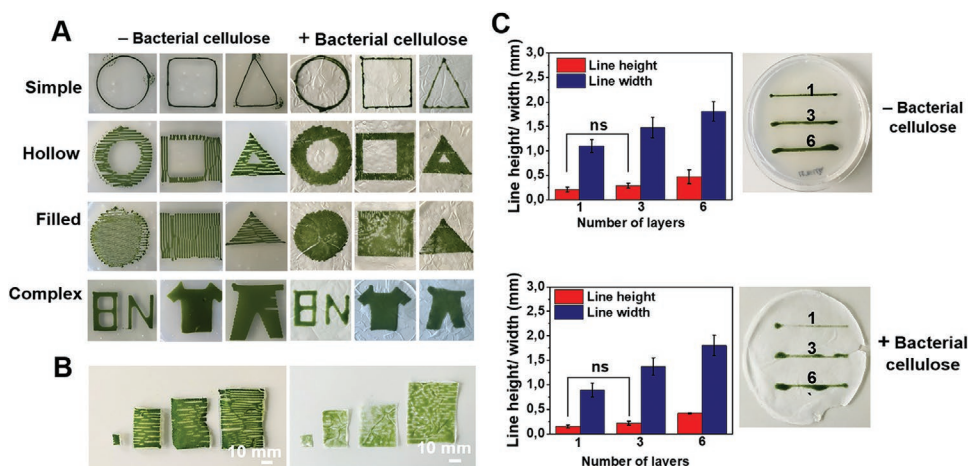


Figure 4. Different geometries and printing resolution of bioprinted microalgal materials. A) Varying geometries (basic: mono-layered structure; complex: multi-layered (2–4 layer) structures) and B) sizes of bioprinted microalgae on carbon-supplemented agar (left) or bacterial cellulose (right). Images were taken after 7 days of photomixotrophic growth. C) Line height and width of bioprinted structures (mono-, tri-, and hexa-layered) on carbon-supplemented agar or bacterial cellulose overtop of carbon-supplemented agar. Measurements were performed after 7 days of photomixotrophic growth ($n = 9$). ns, not significant; all the other comparisons were statistically significant ($p < 0.01$ as determined by one-way (single factor) ANOVA with post-hoc Tukey's HSD).

containing carboxymethyl cellulose, or as bioprints on carbon-supplemented agar containing carboxymethyl cellulose, or as bioprints on bacterial cellulose overtop of carbon-supplemented agar (Figure S7, Supporting Information). In these assays, active digestion of carboxymethyl cellulose in the agar plates is indicated by a halo formation (cellulolytic zone) around the wells due to the enzymatic secretion into the solid medium. Microalgae grown as planktonic cultures expressed cellulolytic activities on carboxymethyl cellulose (18.36 ± 0.27 mm), whereas no cellulolytic zones were observed for bioprinted microalgae on either carboxymethyl cellulose or bacterial cellulose. The lack of cellulolytic zones seen for bioprinted microalgae could be due to the alginate matrix immobilizing secreted enzymes within the bioprinted structures, which would be beneficial from a material perspective since the bacterial cellulose printing substrate would not be digested over time by the microalgae within the bioprints.

2.2. Printability of *C. reinhardtii* with Defined Patterns and Printing Resolution

To characterize the printability of our photosynthetic living material, *C. reinhardtii* were bioprinted in varying pre-defined basic geometrical patterns (simple, hollow, and filled) as single-layered constructs or as multi-layered complex geometrical patterns (2–4 layered) in a range of sizes on carbon-supplemented agar or bacterial cellulose overtop of carbon-supplemented agar. Samples were visually inspected after 7 days of photomixotrophic growth (Figure 4A,B). Microalgae could be bioprinted in all the pre-defined patterns and sizes ($5 \text{ mm} \times 8 \text{ mm}$; $22 \text{ mm} \times 32 \text{ mm}$; $31 \text{ mm} \times 42 \text{ mm}$; $37 \text{ mm} \times 52 \text{ mm}$) both onto carbon-supplemented agar or bacterial cellulose overtop of carbon-supplemented agar. Thus, with our approach we can make at-scale bioprints by merely changing the bioprinting design parameters. Microalgae bioprinted onto bacterial cellulose could be readily peeled off from the agar support layer (Figure S8, Supporting Information), allowing the material to be self-standing, thus considerably increasing its range of possible applications.

The printing resolution for microalgal materials bioprinted with our house-built bioprinter was assessed by characterizing the minimum printed line height and width. We created multi-layered structures (up to 6 layers) by depositing bio-ink on top of previously printed layers in a layer-by-layer fashion. The line heights of the bioprinted microalgae on carbon-supplemented agar and bacterial cellulose overtop of carbon supplemented agar increased steadily as the number of bioprinted layers increased. Meanwhile, the line widths of bioprinted microalgae on carbon-supplemented agar and bacterial cellulose overtop of carbon-supplemented agar also increased as the number of bioprinted layers increased (Figure 4C), though proportionally more slowly, likely due to slight spreading of the hydrogel prior to full gelation during the layer-by-layer addition. We confirmed this hypothesis by printing 10- and 20-layer structures onto both carbon-supplemented agar and bacterial cellulose overtop of carbon supplemented agar, which displayed increased line widths (Figure S9, Supporting Information). In summary, our bioprinting strategy can produce bioprints as lines or curves with sharp millimeter-scale resolution for prints up to 6 layers tall, with a decrease in resolution for additional layers. The resolution obtained with this strategy is similar to that obtained with the bioprinting of bacteria previously reported by our groups.^[6e,25] The resolution is sufficiently high for applications such as biogarments, where patterning of microalgae on bacterial cellulose is desirable.^[3b] Applications such as structural materials that would require an increased number of layers at higher resolution, would benefit from further improvements in 3D printing capability.

2.3. Physical Stability and Regenerative Aspects of Bioprinted Living Materials

In addition to removing the bioprinted microalgae living materials from agar, we were also interested in detaching it from the cellulose substrate, as this would potentially enable other types

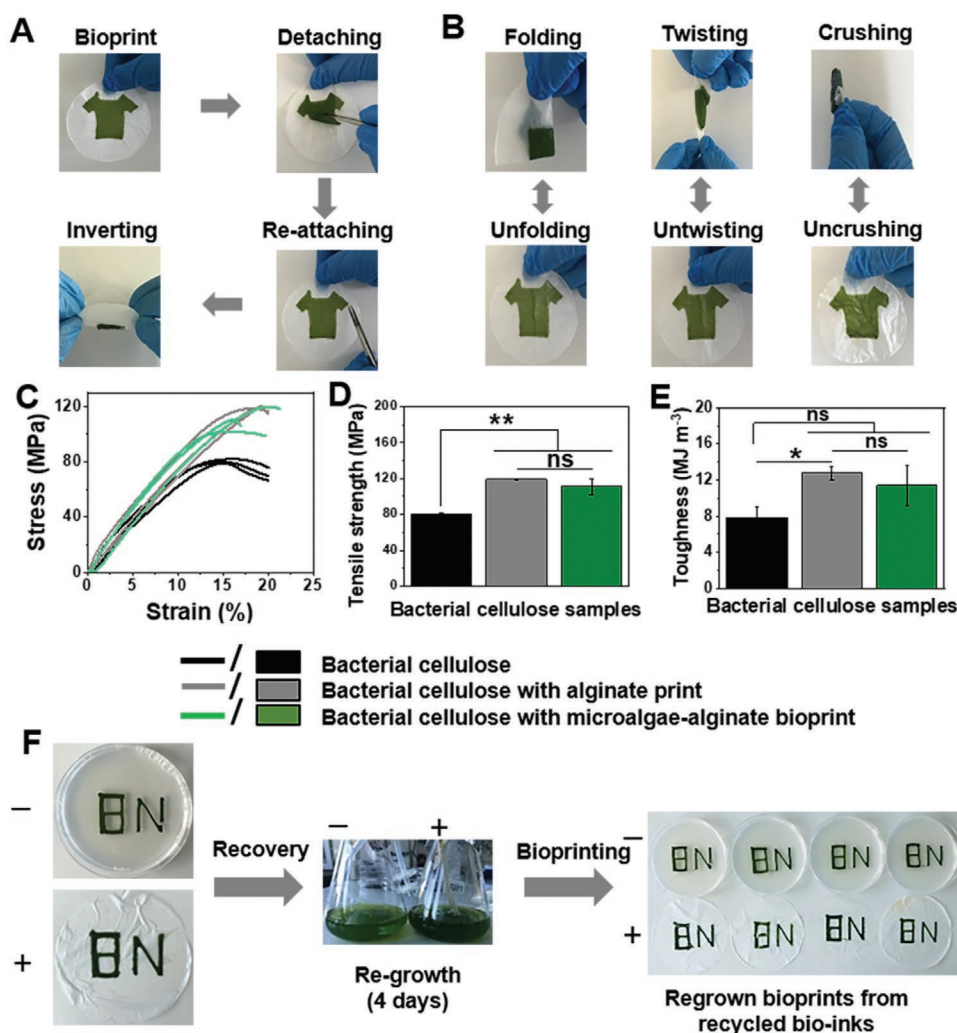


Figure 5. Physical stability and regenerative abilities of the bioprinted living materials. Bioprinted microalgae materials after A) detachment and re-attachment onto bacterial cellulose, and B) physical distortion assessments. Mechanical testing of bioprinted materials including C) tensile stress–strain curves, D) tensile strength, and E) toughness. F) Regeneration from recycled bio-inks. For regeneration, microalgae from one-week-old photomixotrophically grown bioprints on carbon-supplemented agar (–) or bacterial cellulose overtop of carbon-supplemented agar (+) were recovered by citrate treatment. The recovered cells were regrown in carbon-supplemented medium for 4 days, harvested for fresh bio-ink preparation, and bioprinted again. Bioprints were grown for 1 week under photomixotrophic condition and were imaged. ns, not significant; * $p < 0.05$, ** $p < 0.01$ as determined by one-way (single factor) ANOVA with post-hoc Tukey's HSD.

of product applications, for example, as living photosynthetic patterns and brand labels. Upon testing, we found that the microalgal bioprints could successfully be detached from bacterial cellulose and re-attached onto a fresh bacterial cellulose surface. The re-attached bioprints remained attached even after inverting them (Figure 5A).

The stability of microalgal bioprints to physical distortions is also of crucial importance to allow potential user interaction scenarios. We subjected microalgal bioprints on bacterial cellulose to physical distortions manually by folding, twisting, and crushing them at least 6 times each (Figure 5B). The microalgal bioprints on bacterial cellulose all resumed their original shapes upon unfolding, untwisting, and uncrushing, whereas pure alginate prints or microalgae bioprints not supported by bacterial cellulose were fragile and broke within one round of the folding/twisting/crushing assessments due to their poor

mechanical properties (Figure S10, Supporting Information). Thus, the bacterial cellulose support confers excellent mechanical properties to the microalgae bioprints.

To quantify the mechanical properties conferred by bacterial cellulose, we measured the tensile strength and toughness of 1) bacterial cellulose alone, 2) alginate printed onto bacterial cellulose (without microalgae), and 3) microalgae/alginate printed onto bacterial cellulose. The tensile strength of pure bacterial cellulose was 80.3 ± 1.6 MPa, whereas the tensile strength of alginate prints on bacterial cellulose was 119.2 ± 0.9 MPa, and the tensile strength of microalgae bioprints on bacterial cellulose was 110.8 ± 8.9 MPa (Figure 5C,D). The toughness of pure bacterial cellulose was 7.9 ± 1.1 MJ m⁻³, whereas the toughness of alginate prints on bacterial cellulose was 12.8 ± 0.8 MJ m⁻³, and the toughness of microalgae bioprints on bacterial cellulose was 11.4 ± 2.2 MJ m⁻³ (Figure 5E). Thus, the prints on bacterial

cellulose (either alginate or alginate-microalgae bioprints on bacterial cellulose) possessed higher tensile strength and higher toughness than pure bacterial cellulose samples. No significant difference was observed between the tensile strength and toughness of bacterial cellulose supporting prints made of alginate alone versus alginate with microalgae. Therefore, microalgae present in the bioprints did not contribute to the mechanical properties of the living material.

We evaluated the effects on the mechanical properties of the bioprinted living materials of fabrication parameters including elapsed time after printing and number of printed layers (Figure S11, Supporting Information). The tensile strength (Figure S11A,B, Supporting Information) of microalgae printed onto bacterial cellulose increased significantly ($p < 0.05$) from 85.4 ± 8.9 MPa at 0 days after printing to 110.8 ± 8.9 MPa at 7 days after printing, whereas the toughness values remained unchanged over time (Figure S11C, Supporting Information). This increase in tensile strength could be due to increased attachment of the bioprint to the bacterial cellulose over time during the incubation period or to the decrease in water content from evaporative loss of water from the bioprint and bacterial cellulose. Increasing the number of printed layers had no significant effect on the mechanical properties of the bioprinted living materials (Figure S11D–F, Supporting Information). Moreover, bioprints on bacterial cellulose were observed to retain a stable 3D shape and intense green color even after 1 month of storage under ambient conditions (Figure S12, Supporting Information).

The stability of the bioprints in water was assessed by immersing the microalgal bioprints multiple times into water and retrieving them. The bioprints remained stably attached on the bacterial cellulose and did not display observable dissolution even after 6 rounds of immersion in water (Figure S13, Supporting Information). Bioprints on bacterial cellulose were also immersed in water for a period of one week under ambient conditions. Upon retrieval of the immersed samples, no visible distortion of the bioprinted structures was observed. We also tested the mechanical properties of the bioprinted living materials before and after immersion, and after drying the immersed materials (Figure S14, Supporting Information). After immersion in water for one week, the tensile strength of the bioprinted living materials dropped from 110.8 ± 8.9 to 58.2 ± 1.3 MPa. However, when these wet materials were placed out of water for 7 days, the tensile strength increased to 145 ± 12 MPa, which is consistent with our hypothesis that a decrease in water content causes the increase in tensile strength observed in Figure S11, Supporting Information. The values for toughness ranged between 11 and 16 MJ m⁻³ for all the samples without statistically significant differences. Therefore, immersion in water reduces the tensile strength of the bioprinted materials without affecting the toughness. Additionally, no visible presence or growth of microalgae in the water phase was observed during incubation, which was confirmed by measuring the O.D.₇₅₀ of the water samples before immersion and after retrieval of the bioprints (Figure S15, Supporting Information). Thus, no or minimal liberation of microalgae from the bioprints into the water occurred, which is advantageous in applications wherein the bioprints contact water without raising potential environmental concerns.^[26]

The reversible polymerization chemistry of our alginate bio-ink gives rise to the possibility that the bioprinted living materials may be able to be dissolved and re-used. To test this hypothesis, we attempted to dissolve the alginate hydrogel and recover the bioprinted microalgal cells on carbon-supplemented agar or bacterial cellulose otop of carbon-supplemented agar by treatment with sodium citrate (Figure S16, Supporting Information). The recovered microalgal cells were regenerated in fresh carbon-supplemented medium by growth under photo-mixotrophic condition for 4 days. The regenerated microalgal cultures were then used to prepare fresh bio-inks with which several bioprints were made, such that one single print gave rise to multiple prints of the same dimensions, and further grown for 7 days. The fully re-grown bioprinted materials highlight the reusability and regenerative abilities of microalgae in these living materials (Figure 5F). Thus, the fabricated bioprinted living materials can be readily deconstructed following citrate treatment and used as a recycled source for the generation of more of such living materials, indicating their circular nature.^[27]

2.4. Up-Scaled Production of Bioprinted Living Materials

To demonstrate the applicability of this bioprinting approach, we custom-built a second, similar cost-effective bioprinter (Figure S17, Supporting Information) suitable for bioprinting at larger scales (up to 70 cm × 20 cm). We could show that our bioprinted living material (of 22 cm × 12 cm dimension) remained flexible (Figure 6). The flexible nature of bacterial cellulose and the freedom of design achievable with bioprinting technology enable our bioprinted living materials to be used in applications like photosynthetic bio-garments, adhesive labels, and blinds or curtains for windows.

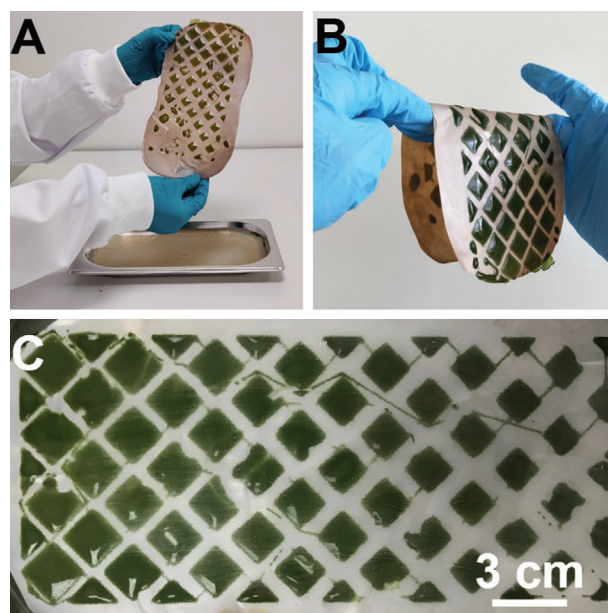


Figure 6. Photographs of up-scaled bioprinted living materials (22 cm × 12 cm) A) Peeled-off from carbon-supplemented agar, B) flexibility of the material, and C) overall view of the living material.

3. Conclusion

The printing of living cells typically involves immobilization or encapsulation of the living cells within a hydrogel matrix. Different hydrogel systems have been shown to be suitable to support cell growth while maintaining the 3D structure,^[1b,4b,6b,7a,c] however, it remains challenging to endow mechanical robustness to bioprinted structures. In this study, we report a calcium-alginate-based hydrogel system for bioprinting of *C. reinhardtii* microalgae cells overtop a cellulose substrate. Calcium-alginate-based hydrogels are easy to pattern and cell-friendly, such that the microalgal cells remain alive both during and after the bioprinting process for relatively long periods of time. Their optical transparency and selective permeability ensure efficient light transmission, mass transfer of nutrients, and diffusion of environmental CO₂ to the bioprinted microalgal cells allowing for vigorous growth. The cross-linked “egg-box” structure of the alginate hydrogel matrix exhibits pore dimensions that enable the retention of microalgal cells, while supporting the release of O₂ produced during the photosynthetic process.^[7b]

The immobilized or encapsulated microalgal cells in the hydrogel matrix of the bioprinted structures retain viability and exhibit increased growth and chlorophyll content over a 4-week period of cultivation. Further, the microalgae in these living materials remain stable in terms of cell density and chlorophyll content for at least a 3-day period after removal from nutrients, and their longevity can be extended by placing them back into contact with nutrients. The fabricated living materials show resilience to physical distortions and to immersion in water. No visible release of the microalgal cells from the bioprinted living materials to surrounding water can be observed, highlighting the biosecurity features of the fabricated living materials that prevent environmental contamination. However, the potential environmental implications of these materials will still need to be closely investigated for individual applications prior to full deployment.

With our approach, we show bioprinted microalgal structures with millimeter-scale precision that support the spatio-temporal control of microalgae. Further, this bioprinting approach is simple, scalable, and eco-friendly, involving usage of completely biodegradable components. Moreover, the costs of the bioprinters are ≈250 US dollars, which is vastly cheaper than the previously reported bioprinters described for microalgal-bioprinting, which have costs ranging from 5000 to 250 000 US dollars.^[7b,c] Thus, the work shown here highlights the development of a cost-effective, efficient, and straight-forward bioprinting approach employing alginate-based chemistry for fabrication of resilient photosynthetic living materials.

There is a growing interest in the development of living materials that are regenerative and reusable in nature.^[27] The regenerative nature of our bioprinted materials can inspire diverse future living products which require curation to extend their life or repair them, with innovative “end-of-life” scenarios.^[3b] For example, if the living materials were physically damaged, the end users could extract the microalgal cells and regrow them into fresh and undamaged bioprinted structures without wasting the original cells in the material. As the bioprinted microalgae are stored within our living materials, they could be directly used as raw materials by the users for their own

production of new living materials at locations unsuitable for new shipments of microalgal cultures, such as for space applications. Such novel user scenarios with living materials should be further explored via user studies in real-life settings.^[3b]

Due to the sustainable nature of our approach, which employs naturally-occurring materials based on living microalgal cells and environmentally friendly biopolymers such as alginate and bacterial cellulose, as well as its physical robustness, resilience, and regenerative nature, the developed bioprinted living materials could be employed in diverse applications including artificial leaves, photosynthetic biogarments, and adhesive labels.

4. Experimental Section

Reagents: Phosphate solution and Hutner's trace elements were obtained from Chlamydomonas Resource Center, USA. All other chemical reagents and solvents (acetic acid, agar, ammonium chloride, calcium chloride, calcium chloride dihydrate, carboxymethyl cellulose, cellulase from *Trichoderma reesei* (aqueous solution, ≥700 units g⁻¹), citric acid, Congo red, dimethyl sulfoxide (DMSO), disodium hydrogen phosphate, glucose, glutaraldehyde, hydrochloric acid, magnesium sulfate heptahydrate, potassium dihydrogen phosphate, sodium alginate, sodium chloride, sodium hydroxide, tris base, tryptone, and yeast extract) were purchased from Sigma Aldrich. Silicone tubing (1 mm × 1 mm) for bioprinting was purchased from VWR international BV.

Strains and Culturing Conditions: *C. reinhardtii* CC-124 wild type mt(–) used in this study was purchased from Chlamydomonas Resource Center, USA. The strain was propagated under laboratory conditions for several weeks to allow for acclimation to humidity and temperature. Cultures were grown in minimal medium (Tris: 2.42 g L⁻¹, TAP salts (NH₄Cl: 5 g L⁻¹, MgSO₄·7H₂O: 4 g L⁻¹, and CaCl₂·2H₂O: 2 g L⁻¹), phosphate solution: 0.03 v/v%, Hutner's trace elements; 0.1 v/v%; pH adjusted to 7.0 with hydrochloric acid) or carbon-supplemented medium (Tris: 2.42 g L⁻¹, TAP salts (NH₄Cl: 5 g L⁻¹, MgSO₄·7H₂O: 4 g L⁻¹, and CaCl₂·2H₂O: 2 g L⁻¹), phosphate solution: 0.03 v/v%, Hutner's trace elements; 0.1 v/v%; pH adjusted to 7.0 with acetic acid) with sterile air bubbling at room temperature. Microalgal bioprints were subjected to light/dark (12:12 h) cycles with a light intensity of 23 μmol m⁻² s⁻¹ using Grow light LEDs strip—Red / Blue—4: 1 (ABC-LED, Netherlands) for a period of 7 days. Spectral data of LEDs (Figure S18, Supporting Information) were obtained with an AQ6374 (350–1750 nm) optical spectrum analyzer.

G. hansenii (ATCC 53582) used for bacterial cellulose production was cultured in Hestrin–Schramm (HS) medium (tryptone: 5.0 g L⁻¹, yeast extract: 5.0 g L⁻¹, disodium hydrogen phosphate: 2.7 g L⁻¹, citric acid: 1.5 g L⁻¹, and glucose: 20 g L⁻¹) statically at 30 °C for 3 to 4 days to obtain a bacterial cellulose pellicle. The inoculum for bacterial fermentation was prepared by treating this bacterial cellulose pellicle with cellulase (0.1 v/v%) with shaking at 180 rpm at 30 °C overnight. This solution was then centrifuged at 4000 rpm for 5 min at 4 °C, and the obtained bacterial pellet was re-suspended in fresh HS medium. A 1 v/v% of this solution was used as the inoculum for bacterial fermentation.

Bacterial Cellulose Production and Purification: Bacterial cellulose was produced in vitro as a pellicle at the air-liquid interface by the static fermentation of *G. hansenii* in HS medium in a Petri dish (94 mm × 16 mm) for 7 days at 30 °C. The produced bacterial cellulose pellicle was submerged in NaOH (1 w/v%) solution and boiled for 10 min to heat-kill the producing *G. hansenii*. The bacterial cellulose pellicle was then washed several times in hot water to remove the impurities and residual HS media components. Finally, the purified bacterial cellulose pellicles were air-dried and autoclaved before their use in experiments with microalgae and bioprinting.

***C. reinhardtii* Growth on Bacterial Cellulose:** To assess microalgal growth on bacterial cellulose, sterile bacterial cellulose samples (1 cm × 1 cm) were submerged aseptically in freshly prepared *C. reinhardtii*

(10^4 cells mL⁻¹) solution and placed onto carbon-supplemented agar medium. Samples were incubated at room temperature and subjected to light/dark (12:12 h) cycles. After 3 and 7 days of incubation, samples were visually inspected and photographed.

Bioprinting Substrate Preparation: To print onto agar surfaces, a Petri dish (94 mm × 16 mm) filled with 25 mL of minimal agar or carbon-supplemented agar with calcium chloride (0.05 M) was used as the printing substrate.

To print onto bacterial cellulose, sterile bacterial cellulose was placed overtop of freshly prepared minimal medium or carbon-supplemented agar medium supplemented with calcium chloride (0.05 M) and attached to this agar surface by addition of 500 µL calcium chloride (5 M). The surface of the bacterial cellulose was flattened with an L-shaped spreader. Plates with bacterial cellulose were sterilized by UV-treatment for 1 h in a laminar flow chamber and then used as the printing substrate.

Bio-Ink Preparation: For the preparation of the bio-ink for bioprinting, 10 mL of a 7-day microalgae liquid culture (grown in carbon-supplemented medium under light/dark condition (14:10 h)^[28] with sterile air bubbling) was spun down at 4000 rpm for 5 min, and the supernatant was discarded. Cells were re-suspended in 10 mL of minimal medium or carbon-supplemented medium. An equal volume of sodium alginate (5 w/v%) was added to the microalgal cell suspension and vortexed to obtain the bio-ink for the printing process. The final microalgal cell concentration was 1×10^6 cells mL⁻¹, and the sodium alginate concentration in the bio-ink was 2.5 w/v%.

Bioprinting: Bioprinting was performed using a modified do-it-yourself bioprinter (CoLiDo DIY) as previously described.^[6e,25a] Briefly, the extruder and heater of the standard bioprinter were removed and replaced with a 0.2 mL pipette tip, a silicone tubing system, and a syringe pump (Figure S1, Supporting Information). Desired 3D structures with different shapes and sizes were designed in CoLiDo software by manually programming the G-codes. During the bioprinting, a sterile syringe was loaded with 10 mL of printer bio-ink and mounted in a syringe pump. The syringe was connected to a 0.2 mL pipette tip via silicone tubing (1 mm × 1 mm). Printing substrates were placed onto the stage, and printing was then carried out using the CoLiDo software interface. An extrusion rate of 0.5 mL h⁻¹ was maintained throughout the process of bioprinting.

Methods associated with the characterization of the bioprints can be found in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author contributions

S.B. and K.Y. contributed equally to this work. All authors conceived and designed the experiments. S.B. and K.Y. performed the experiments and analyzed the data. All authors prepared and revised the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

3D printing, bacterial cellulose, biofabrication, *Chlamydomonas reinhardtii*, hydrogel

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